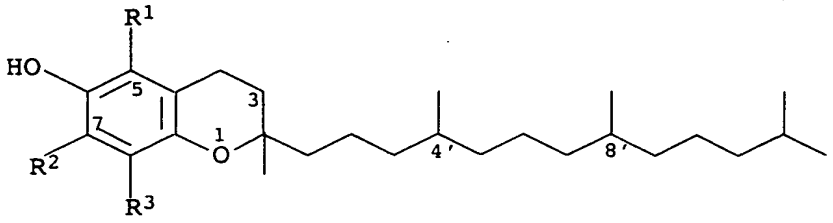
5/prts

Homogentisate dioxygenase

The present invention relates to novel genetic constructs such as expression cassettes and vectors for generating plants with an elevated tocopherol content, to transgenic plants generated thus, and to methods for the generation of transgenic plants with an elevated tocopherol content.

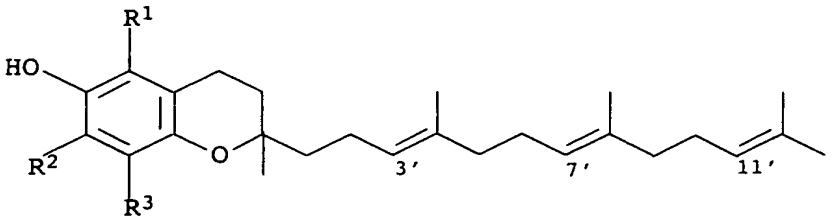
The generation of plants with an elevated sugar, enzyme and amino acid content has hitherto been an important objective in plant molecular genetics. The development of plants with an elevated vitamin content, such as, for example, with an elevated tocopherol (vitamin E) content, is, however, also of economic interest.

The naturally occurring eight compounds with vitamin E activity are derivatives of 6-chromanol (Ullmann's Encyclopedia of Industrial Chemistry, Vol. A 27 (1996), VCH Verlagsgesellschaft, Chapter 4., 478-488, Vitamin E). The first group (1a-d) encompasses the tocopherols (I), while the second group (2a-d) encompasses the tocotrienols (II):



(I)

1a, α-tocopherol: $R^1 = R^2 = R^3 = CH_3$
1b, β-tocopherol: $R^1 = R^3 = CH_3$, $R^2 = H$
1c, γ-tocopherol: $R^1 = H$, $R^2 = R^3 = CH_3$
1d, δ-tocopherol: $R^1 = R^2 = H$, $R^3 = CH_3$



(II)

2a, α-tocopherol: $R^1 = R^2 = R^3 = CH_3$
2b, β-tocopherol: $R^1 = R^3 = CH_3$, $R^2 = H$
2c, γ-tocopherol: $R^1 = H$, $R^2 = R^3 = CH_3$
2d, δ-tocopherol: $R^1 = R^2 = H$, $R^3 = CH_3$

where
R^1, R^2 and R^3 are as defined above.

At present, alpha-tocopherol is of great economic importance.

The development of crop plants with an elevated tocopherol content by means of tissue culture or seed mutagenesis and natural selection is set a limit. On the one hand, it must be possible for the tocopherol content to be recorded as early as during the tissue culture stages and, on the other hand, only those plants can be manipulated via tissue culture techniques which can be successfully regenerated from cell cultures into whole plants. Moreover, crop plants can show undesirable properties after mutagenesis and selection, and the former have to be eliminated by in some cases repeated backcrosses. Also, increasing the tocopherol content by means of crosses would be limited to plants of the same species.

This is why the genetic engineering approach of isolating the essential biosynthesis genes which encode tocopherol synthesis performance and introducing them into crop plants in a directed fashion is superior to the traditional plant breeding method. Knowledge of the biosynthesis pathways and its regulation, and identification of genes which affect biosynthesis performance, are prerequisites for this method.

The tocopherol synthesis pathway in plants is shown schematically in the appended Figure 1. As yet, no useful approach exists in the prior art which allows the tocopherol biosynthesis in plants to be elevated in a targeted fashion.

Short description of the invention:

It is an object of the present invention to provide means with the aid of which an improved tocopherol biosynthesis can be achieved.

We have found that this object is achieved by providing genetic constructs with the aid of which the biosynthesis of homogentisate, a tocopherol precursor, and thus the formation of tocopherol, can be increased. Simultaneously, it is possible in accordance with the invention to prevent the undesired homogentisate efflux to maleyl acetoacetate, thus improving tocopherol synthesis further.

A first subject of the invention therefore relates to an expression cassette comprising

a) the coding nucleic acid sequence for 4-hydroxyphenylpyruvate dioxygenase (HPPD) or for a functional equivalent thereof, thus increasing the homogentisate biosynthesis rate upon expression; and/or

b) at least one nucleic acid sequence (anti-HGD) which is capable of inhibiting the homogentisate dioxygenase (HGD) activity

under the genetic control of regulatory nucleic acid sequences.

"Inhibition" is to be interpreted broadly in the present context and encompasses the partial or essentially complete prevention or blocking of the HGD enzyme activity in the plant or the plant organ or tissue which has been transformed with an anti-HGD construct according to the invention, this prevention or blocking being based on different mechanisms in cell biology. Inhibition for the purposes of the invention also encompasses a quantitative reduction of active HGD in the plant down to an essentially complete absence (i.e. lack of detectability of HGD enzyme activity or lack of immunological detectability of HGD) of HGD protein.

According to the invention several strategies for decreasing or inhibiting of the HGD activity are comprised. A person skilled in the art will recognize that a variety of different methods are available in order to affect the HGD gene expression in a desirable manner.

A preferred strategy according to the invention comprises the use of a nucleic acid sequence (anti-HGD) which can be transcribed into an antisense nucleic acid sequence which is capable of inhibiting the homogentisate dioxygenase (HGD) activity, for example by inhibiting the expression of endogenous HGD.

Further methods of inhibiting the HGD expression comprise the overexpression of homologous HGD nucleic acid sequences leading to cosuppression (Jorgensen et al. (1996): "Chalcone synthase cosuppression phenotypes in petunia flowers: Comparison of sense vs. antisense constructs and single copy vs. complex T-DNA sequences.", Plant Mol. Biol. 31 (5): 957-973.), induction of specific RNA degradation by a plant with the help of a viral expression system (amplicon) (Angell, S. M., Baulcombe, D. C. (1999): "Technical advance: Potato virus x amplicon mediated silencing of nuclear genes." Plant J. 20 (3): 357-362.),

insertion of nonsense mutations into the endogene by means of introduction of RNA/DNA oligo nucleotides into the plant (Zhu et al. (2000): "Engineering herbicide resistant maize using chimeric RNA/DNA oligonucleotides." Nat. Biotechnol. 18 (5): 555-558.) or generating knockout mutants, e. g. with the help of T-DNA mutagenesis (Koncz et al. (1992): "T-DNA insertional mutagenesis in Arabidopsis." Plant Mol. Biol. 20 (5): 963-976.) or homolgous recombination (Hohn, B.; Puchta, H. (1999): "Gene therapy in plants." Proc. Natl. Acad. Sci. USA 96: 8321-8323.).

The above mentioned documents and the methods for the regulation of gene expression in plants described therein are herewith incorporated by reference.

The anti-HGD sequence in the sense of the present invention is thus particularly selected among:

a) antisense nucleic acid sequences;
b) nucleic acid sequences coding for homologous HGD and leading to cosuppression;
c) viral nucleic acid sequences and expression constructs affecting HGD-RNA degradation;
d) nonsense mutations of nucleic acid sequences coding for endogenous HGD;
e) nucleic acid sequences coding for knockout mutants;
f) nucleic acid sequences suitable for homologous recombination;

wherein the expression of each of these sequences can effect an "inhibition" of the HGD activity in the sense of the present invention. A combined use of such sequences is also possible.

The coding HPPD sequence is according to the invention preferably linked functionally to the coding sequence of a plant-organelle-specific transit peptide. The transit peptide preferably has specificity for the seeds or the plastids such as, for example, the chloroplasts, chromoplasts and/or leukoplasts, of the plant. The transit peptide directs the expressed HPPD activity to the desired target within the plant and, once this is achieved, is eliminated from the HPPD protein moiety, preferably proteolytically. In the expression construct according to the invention, the coding transit peptide sequence is preferably located 5'-upstream from the coding HPPD sequence.

In a further preferred embodiment, the coding HPPD sequence and the anti-HGD sequence are in each case under the genetic control of a plant-specific promoter.

Expression cassettes which are especially preferred in accordance with the invention encompass a coding HPPD nucleic acid sequence which encodes a protein containing an amino acid sequence in accordance with SEQ ID NO:15 or a functional equivalent thereof, or which encompasses a nucleic acid sequence from the nucleotide in position 8 to the nucleotide in position 1153 inclusive, in accordance with SEQ ID NO:14 or a functional equivalent thereof.

In a preferred embodiment the anti-HGD nucleic acid sequence can contain the coding nucleic acid sequence of homogentisate dioxygenase or a functional fragment thereof, inserted in antisense orientation. A preferred embodiment of the expression cassettes according to the invention encompasses an HGD sequence motif in accordance with SEQ ID NO:1 in antisense orientation. This leads to the increased transcription of nucleic acid sequences in the transgenic plant which are complementary to the endogenous coding HGD sequence or a portion thereof and which hybridize herewith at DNA or RNA level.

The invention further relates to recombinant vectors encompassing at least one expression cassette in accordance with the above definition. Examples of vectors according to the invention encompass at least one expression construct of the following type:

5'-plant-specific promoter/HPPD or anti-HGD/terminator-3'. The coding HPPD sequence may also be replaced by a coding sequence for a fusion protein of transit peptide and HPPD.

Preferred examples encompass monomeric vectors comprising one of the following expression constructs:

a) 5'-35S-promoter/anti-HGD/OCS-terminator-3';
b1) 5'-legumin-B-promoter/HPPD/NOS-terminator-3';
b2) 5'-legumin-B-promoter/transit peptide-HPPD/NOS-terminator-3'.

The constructs a) and b) require the plant to be co-transformed with both vectors, i.e. with a) and b1) or b2).

Preferred examples also encompass binary vectors comprising the following constructs:

c1) 5'-35S-promoter/anti-HGD/OCS-terminator/legumin-B-promoter/HPPD/NOS-terminator-3'; and
c2) 5'-35S-promoter/anti-HGD/OCS-terminator/legumin-B-promoter/transit peptide-HPPD/NOS-terminator-3'.

Construct c1) or c2) allows the simultaneous transformation of the plant with HPPD and anti-HGD.

The invention furthermore relates to microorganisms comprising at least one recombinant vector according to the invention. Preferred organisms are those which are capable of infecting plants and thus of transferring the constructs according to the invention.

Preferred microorganisms are those from the genus Agrobacterium, in particular the species Agrobacterium tumefaciens.

The invention furthermore relates to the use of a vector or microorganism according to the invention for the transformation of plants, plant cells, plant tissues or plant organs, in particular with the purpose of making them capable of an improved tocopherol synthesis.

The invention furthermore relates to a transgenic plant, transformed with at least one vector or microorganism according to the invention, and to transgenic cells, tissue, organs or transgenic propagation material of such plants.

The transgenic plants according to the invention are in particular selected from amongst crop plants such as cereals, maize, soybeans, rice, cotton, sugar beet, canola, sunflowers, flax, potatoes, tobacco, tomatoes, oilseed rape, alfalfa, salad species such as cress, and the various tree, nut and grapevine species.

The invention furthermore relates to a method for generating transgenic plants with improved tocopherol production, which comprises transforming plants which are capable of producing tocopherol, or plant cells, plant tissue or plant organs or protoplasts thereof, with at least one vector according to the invention or at least one microorganism according to the invention, culturing the transformed cells, tissue, plant organs or protoplasts in a growth medium and, if appropriate, regenerating plants from the culture.

The invention furthermore relates to the use of an expression cassette, a vector, a microorganism or a transgenic plant in accordance with the above definition for obtaining plant metabolites, in particular tocopherols.

Finally, the invention relates to a process for the preparation of tocopherols, which comprises isolating in a known manner the desired tocopherol from a culture of a plant which has been transformed in accordance with the invention.

Detailed description of the invention:

The transformation according to the invention of plants with an HPPD-encoding construct leads to the overexpression of this protein and thus to an increased homogentisate formation. The simultaneous transformation with anti-HGD, in particular the antisense-HGD construct, avoids an undesired efflux of this metabolite to maleyl acetoacetate. Thus, an increased homogentisate quantity is available in the transgenic plant for the formation of tocopherols via the intermediates methyl-6-phytylquinol and 2,3-dimethylphytylquinol (cf. Figure 1).

A nucleotide or nucleic acid sequence is to be understood as meaning in accordance with the invention for example a genomic or a complementary DNA sequence or an RNA sequence or semi- or fully synthetic analogs thereof.

The HPPD or anti-HGD nucleotide sequences of the constructs according to the invention can be produced synthetically or obtained naturally or comprise a mixture of synthetic and natural DNA components, or else be composed of various heterologous HGD or HPPD gene segments of various organisms. The anti-HGD sequence can be derived from one or more exons and/or introns, in particular exons of the HGD gene.

For example, synthetic nucleotide sequences can be generated which have codons which are preferred by the plants to be transformed. These codons which are preferred by plants can be determined for the plant in the customary manner with the aid of the codon usage. When preparing an expression cassette, various DNA fragments can be manipulated in such a way that the result is a nucleotide sequence with the correct direction of reading and a correct reading frame. To connect the nucleic acid fragments to each other, adapters or linkers may be attached to the fragments.

Functional equivalents of the HPPD gene are those sequences which still encode a protein with the desired functions in accordance with the invention, i.e. an enzyme with homogentisate-forming activity, despite a deviating nucleotide sequence.

Functional equivalents of anti-HGD encompass those nucleotide sequences which prevent the HGD enzyme function in the transgenic plant to a sufficient degree. This can be effected for example by hindering or preventing HGD processing, the transport of HGD or its mRNA, inhibition of ribosome attachment, inhibition of RNA splicing, induction of an RNA-degrading enzyme and/or inhibition of translation elongation or translation termination.

Functional equivalents generally encompass naturally occuring variants of the sequences described herein and also artificial nucleotide sequences, for example artificial nucleotide sequences obtained by chemical synthesis which are adapted to the codon usage of a plant.

Functional equivalents are also to be understood as meaning, in particular, natural or artificial mutations of an originally isolated sequence which encodes HGD or HPPD which continue to show the desired function. Mutations encompass substitutions, additions, deletions, exchanges or insertions of one or more nucleotide residues. Thus, the present invention also encompasses, for example, those nucleotide sequences which are obtained by modifying the HGD or HPPD nucleotide sequence. The purpose of such a modification may be, for example, the further limitation of the encoding sequence contained therein or else, for example, the insertion of further restriction enzyme cleavage sites.

Functional equivalents are also those variants whose function is attenuated or increased compared with the starting gene or gene fragment, that is to say for example those HPPD genes which encode an HPPD variant with a lower or higher enzymatic activity than that of the original gene.

Also suitable are artificial nucleic acid sequences as long as they mediate the desired characteristic, for example an elevated tocopherol content in the plant, by overexpression of the HPPD gene or expression of an anti-HGD sequence in crop plants, as described above. Such artificial nucleotide sequences can be identified, for example, by back translation of proteins with HGD or HPPD activity which have been constructed by means of molecular modeling, or else by in vitro selection. Especially suitable are coding nucleotide sequences which have been obtained by back translating a polypeptide sequence in accordance with the host-plant-specific codon usage. An expert skilled in the art of plant genetic methods will readily be able to identify the specific codon usage by computer evaluations of other known genes of the plant to be transformed. To circumvent undesired

regulatory mechanisms of the plant, it is possible, for example, to back translate DNA fragments starting from the amino acid sequence of a bacterial HPPD and taking into consideration the plant codon usage, and thus generate the complete exogenous HPPD sequence which is optimized for use in the plant. This expresses an HPPD enzyme which is not, or only insufficiently, accessible to regulation by the plant, thus fully allowing enzyme activity to be overexpressed.

Further suitable equivalent nucleic acid sequences which must be mentioned are sequences which encode fusion proteins, for example an HPPD polypeptide or a functionally equivalent portion of these being a constituent of the fusion protein. The second portion of the fusion protein may be, for example, another enzymatically active polypeptide, or an antigenic polypeptide sequence with the aid of which detection of HPPD expression is possible (for example myc-tag or his-tag). However, it is preferably a regulatory protein sequence such as, for example, a signal or transit peptide which leads the HPPD protein to the desired site of action.

An elevated tocopherol content in the plant is to be understood as meaning for the purposes of the present invention the artificially acquired capability of an increased biosynthetic performance regarding at least one compound from the group of the tocopherols and tocotrienols as defined above in the plant in comparison with the non-genetically-modified plant for the duration of at least one plant generation.

The tocopherol biosynthesis site is generally the leaf tissue but also the seed, so that leaf-specific and/or seed-specific expression in particular of the HPPD gene and/or, if appropriate, anti-HGD are meaningful. However, it is obvious that tocopherol biosynthesis need not be limited to the seed but may also take place in a tissue-specific fashion in all the other remaining parts of the plant.

Constitutive expression of the exogenous gene is also advantageous. On the other hand, inducible expression may also be desirable.

The regulatory nucleic acid sequences contained in the expression cassettes according to the invention govern the expression of the coding sequences (such as HPPD sequence, if appropriate fused to a transit peptide sequence) and the anti-HGD sequence. Preferably, the constructs according to the invention comprise a promoter 5'-upstream from the coding sequence in question and a

terminator sequence 3'-downstream, and, if appropriate, other customary regulatory elements, in each case operatively linked with the coding sequence. Operative linkage is to be understood as meaning the sequential arrangement of promoter, encoding sequence, terminator and, if appropriate, further regulatory elements in such a way that each of the regulatory elements can fulfill its function as intended when the encoding sequence or the antisense sequence is expressed. Examples of operatively linkable sequences are other targeting sequences (which differ from the transit-peptide-encoding sequences) for guaranteeing subcellular localization in the apoplast, in the vacuole, in plastids, in the mitochondrion, in the endoplasmatic reticulum (ER), in the nucleus, in elaioplasts or in other compartments; and translation enhancers such as the tobacco mosaic virus 5'-leader sequence (Gallie et al., Nucl. Acids Res. 15 (1987), 8693 -8711), and the like.

Suitable polyadenylation signals are plant polyadenylation signals, preferably those which essentially correspond to Agrobacterium tumefaciens T-DNA polyadenylation signals, in particular those of gene 3 of the T-DNA (octopine synthase) of the Ti plasmid pTiACHS (Gielen et al., EMBO J. 3 (1984), 835 et seq.) or functional equivalents thereof. Examples of especially suitable terminator sequences are the OCS (octopine synthase) terminator and the NOS (nopaline synthase) terminator.

A suitable promoter of the expression cassettes is, in principle, any promoter which is capable of governing the expression of genes, in particular foreign genes, in plants. In particular, a plant promoter or a promoter derived from a plant virus is preferably used. Particularly preferred is the cauliflower mosaic virus CaMV 35S promoter (Franck et al., Cell 21 (1980), 285 - 294). As is known, this promoter contains various recognition sequences for transcriptional effectors which in their totality lead to permanent and constitutive expression of the introduced gene (Benfey et al., EMBO J. 8 (1989), 2195-2202). Another example of a suitable promoter is the legumin B promoter (accession No. X03677).

The expression cassette may also comprise a chemically inducible promoter which allows expression of the exogenous gene in the plant to be governed at a particular point in time. Such promoters, for example the PRP1 promoter (Ward et al., Plant. Mol. Biol. 22 (1993), 361-366), a salicylic-acid-inducible promoter (WO 95/19443), a benzenesulfonamide-inducible promoter (EP-A-0388186), a tetracyclin-inducible promoter (Gatz et al., (1992) Plant J. 2, 397-404), an abscisic-acid-inducible promoter

(EP-A 335528) or an ethanol- or cyclohexanone-inducible promoter (WO 93/21334), may also be used.

Furthermore, particularly preferred promoters are those which ensure expression in tissues or plant organs in which the biosynthesis of tocopherol or its precursors takes place. Promoters which ensure leaf-specific expression must be mentioned in particular. Promoters which must be mentioned are the potato cytosolic FBPase or the potato ST-LSI promoter (Stockhaus et al., EMBO J. 8 (1989), 2445 - 245). Examples of seed-specific promoters are the phaseolin promoter (US 5504200), the USP promoter (Baumlein, H. et al., Mol. Gen. Genet. (1991) 225 (3), 459 - 467) or the LEB4 promoter (Fiedler, U. et al., Biotechnology (NY) (1995), 13 (10) 1090) together with the LEB4 signal peptide.

An expression cassette is generated by fusing a suitable promoter to a suitable anti-HDG or HPPD nucleotide sequence, if appropriate a sequence encoding a transit peptide, which is preferably arranged between the promoter and the HPPD sequence, and a terminator or polyadenylation signal. To this end, customary recombination and cloning techniques are used as they are described, for example, by T. Maniatis, E.F. Fritsch and J. Sambrook, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989) and by T.J. Silhavy, M.L. Berman and L.W. Enquist, Experiments with Gene Fusions, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1984) and by Ausubel, F.M. et al., Current Protocols in Molecular Biology, Greene Publishing Assoc. and Wiley Interscience (1987).

As already mentioned other expression cassettes which can be used are those whose DNA sequence encodes an HPPD fusion protein, part of the fusion protein being a transit peptide which governs translocation of the polypeptide. Examples are chloroplast-specific transit peptides which are cleaved off enzymatically from the HPPD residue after translocation of the HPPD gene into the chloroplasts.

Particular mention must be made of the transit peptide derived from plastid transketolase (TK) or from a functional equivalent of this transit peptide (for example the transit peptide of the RubisCO small subunit or of ferredoxin: NADP oxidoreductase).

The promoter and terminator regions may expediently be provided, in the direction of transcription, with a linker or polylinker containing one or more restriction sites for insertion of this

sequence. As a rule, the linker has 1 to 10, in most cases 1 to 8, preferably 2 to 6, restriction sites. In general, the linker within the regulatory regions has a size less than 100 bp, frequently less than 60 bp, but at least 5 bp.

The promoter, terminator and the other regulatory elements may be native (homologous) or else foreign (heterologous) to the host plant.

Genetic manipulations which provide suitable restriction cleavage sites or which eliminate the excess DNA or restriction cleavage sites may also be employed for the purposes of the invention. Techniques known per se, such as, in-vitro mutagenesis, primer repair, restriction or ligation may be used in cases where insertions, deletions or substitutions such as, for example, transitions and transversions, are suitable. Complementary ends of the fragments may be provided for ligation by manipulations such as, for example, restriction, chewing back or filling in overhangs for blunt ends.

The expression cassettes according to the invention are preferably inserted into suitable transformation vectors. Suitable vectors are described, inter alia, in "Methods in Plant Molecular Biology and Biotechnology" (CRC Press), Chapter 6/7, pp. 71 - 119 (1993).

They are preferably cloned into a vector such as, for example, pBin19, pBinAR, pPZP200 or pPTV, which is suitable for transforming Agrobacterium tumefaciens. Agrobacteria transformed with such a vector can then be used in a known manner for transforming plants, in particular crop plants, such as, for example, tobacco plants, for example by bathing wounded leaves or leaf sections in an agrobacterial suspension and subsequently growing them in suitable media. The transformation of plants by agrobacteria is known, inter alia, from F.F. White, Vectors for Gene Transfer in Higher Plants; in Transgenic Plants, Vol. 1, Engineering and Utilization, edited by S.D. Kung and R. Wu, Academic Press, 1993, pp. 15 - 38.

Transgenic plants can be regenerated in a known manner from the transformed cells of the wounded leaves or leaf sections.

The transfer of foreign genes into the genome of a plant is termed transformation. It exploits the above-described methods of transforming and regenerating plants from plant tissues or plant cells for transient or stable transformation. Suitable methods are protoplast transformation by polyethylene-glycol-induced DNA

uptake, the biolistic method using the gene gun, the so-called particle bombardment method, electroporation, incubation of dry embryos in DNA-containing solution, microinjection and agrobacterium-mediated gene transfer. The abovementioned methods are described, for example, in B. Jenes et al., Techniques for Gene Transfer, in: Transgenic Plants, Vol. 1, Engineering and Utilization, edited by S.D. Kung and R. Wu, Academic Press (1993), 128 - 143, and in Potrykus, Annu. Rev. Plant Physiol. Plant Molec. Biol. 42 (1991), 205 - 225). The construct to be expressed is preferably cloned into a vector which is suitable for the transformation of Agrobacterium tumefaciens, for example pBin19 (Bevan et al., Nucl. Acids Res. 12 (1984), 8711).

Agrobacteria transformed with an expression cassette can equally be used in a known manner for transforming plants, in particular crop plants such as cereals, maize, oats, soybeans, rice, cotton, sugar beet, canola, sunflowers, flax, hemp, potatoes, tobacco, tomatoes, oilseed rape, alfalfa, lettuce and the various tree, nut and grapevine species, for example by bathing wounded leaves or leaf sections in an agrobacterial suspension and subsequently growing them in suitable media.

The invention also relates to transgenic plants transformed with an expression cassette according to the invention and to transgenic cells, tissue, organs and propagation material of such plants. Especially preferred in this context are transgenic crop plants such as, for example, barley, wheat, rye, maize, oats, soybeans, rice, cotton, sugar beet, canola, sunflowers, flax, hemp, potatoes, tobacco, tomatoes, oilseed rape, alfalfa, lettuce and the various tree, nut and grapevine species. Plants for the purposes of the invention are monocotyledonous and dicotyledonous plants or algae.

The invention is now illustrated in greater detail in the use examples which follow, taking into consideration the appended figures:

Figure 1 shows a schematic representation of the tocopherol biosynthesis pathway in plants; PP represents pyrophosphate; if homogentisate is reacted with geranylgeranyl-PP (not shown) in the plant, the corresponding tocotrienols are formed in a similar fashion;

Figure 2 shows a binary transformation vector which expresses HPPDop in the seeds of transformed plants and simultaneously suppresses the expression of the

endogenous HGD: A = 35S-promoter; B = HGD in antisense orientation; C = OCS terminator; D = legumin B promoter; E = FNR transit peptide; F = HPPDop; G = NOS terminator;

Figure 3 shows construction schemes of the HPPD-encoding plasmids pUC19HPPDop and pCRScriptHPPDop;

Figure 4 shows construction schemes of the antiHGD-encoding plasmids pBinARHGDanti and pCRScriptHGDanti; and

Figure 5 shows construction schemes of the transformation vectors pPTVHGDanti and pPZP200HPPD.

General methods:

a) General cloning methods

The cloning steps carried out for the purposes of the present invention, such as, for example, restriction cleavages, agarose gel electrophoresis, purification of DNA fragments, transfer of nucleic acids to nitrocellulose and nylon membranes, linkage of DNA fragments, transformation of E. coli cells, growing bacteria, multiplying phages and sequence analysis of recombinant DNA, were carried out as described by Sambrook et al. (1989) Cold Spring Harbor Laboratory Press; ISBN 0-87969-309-6.

b) Sequence analysis of recombinant DNA

The recombinant DNA molecules were sequenced with a Licor laser fluorescence DNA sequencer (available from MWG Biotech, Ebersbach) by the method of Sanger (Sanger et al., Proc. Natl. Acad. Sci. USA 74 (1977), 5463 - 5467).

Example 1: Cloning a hydroxyphenylpyruvate dioxygenase (HPPD) with a DNA sequence optimized for expression in *Brassica napus*

The amino acid sequence of the hydroxyphenylpyruvate dioxygenase (HPPD) from *Streptomyces avermitilis* (accession No. U11864) was back-translated into a DNA sequence taking into consideration the codon usage in *Brassica napus* (oilseed rape). The codon usage was determined by means of the database http://www.dna.affrc.go.jp/~nakamura/index.html. The deduced sequence (SEQ ID NO:14) was synthesized by ligating overlapping oligonucleotides, followed by PCR amplification (Rouwendal, GJA; et al, (1997) PMB 33: 989-999), while attaching SalI cleavage sites. The correctness of the sequence of the synthetic gene was checked by sequencing. The

synthetic gene was inserted into the vector pBluescript II SK+ (Stratagene).

Example 2: Cloning a *Brassica napus* homogentisate dioxygenase (HGD)

a) Isolation of total RNA from *Brassica napus* flowers

Open flowers were harvested from *Brassica napus* var. Westar and frozen in liquid nitrogen. The material was subsequently reduced to a powder in a mortar and taken up in Z6 buffer (8M guanidinium hydrochloride, 20 mM MES, 20 mM EDTA, brought to pH 7.0 with NaOH; treated with 400 µl mercaptoethanol/100 ml buffer immediately prior to use). The suspension was then transferred into reaction vessels and extracted by shaking with one volume of phenol/chloroform/isoamyl alcohol 25:24:1. After centrifugation for 10 minutes at 15,000 rotations, the supernatant was transferred into a fresh reaction vessel and the RNA was precipitated with 1/20 volume 1N acetic acid and 0.7 volume ethanol (absolute). After recentrifugation, the pellet was first washed with 3M sodium acetate solution and, after a further centrifugation, with 70% ethanol. The pellet was subsequently dissolved in DEPC (diethylpyrocarbonate) water, and the RNA concentration was determined photometrically.

b) Preparation of cDNA from total RNA from *Brassica napus* flowers

First, 20 µg of total RNA were treated with 3.3 µl of 3M sodium acetate solution, 2 µl of 1M magnesium sulfate solution and made up to an end volume of 10 µl with DEPC water. To this, 1 µl of RNase-free DNase (Boehringer Mannheim) was added, and the mixture was incubated for 45 minutes at 37 degrees. After the enzyme had been removed by extraction by shaking with phenol/chloroform/isoamyl alcohol, the RNA was precipitated with ethanol and the pellet was taken up in 100 µl of DEPC water. 2.5 µg of RNA from this solution were transcribed into cDNA using a cDNA kit (Gibco BRL) following the manufacturer's instructions.

c) PCR amplification of a subfragment of the *Brassica napus* HGD

A comparison of the DNA sequences of the known homogentisate dioxygenases (HGD) from Arabidopsis thaliana (accession No. U80668), Homo sapiens (accession No. U63008) and Mus musculus (accession No. U58988) allowed oligonucleotides to be deduced, for a PCR, which had an SalI cleavage site added at the 5' terminus and an Asp718 restriction cleavage site at the 3'

terminus. The oligonucleotide at the 5′ terminus encompasses the sequence:

GTCGACGGNCCNATNGGNGCNAANGG (SEQ ID NO:2),

starting with base 661 of the Arabidopsis gene. The oligonucleotide at the 3′ terminus encompasses the sequence:

GGTACCTCRAACATRAANGCCATNGTNCC (SEQ ID NO:3),

starting with base 1223 of the Arabidopsis gene, where N is in each case inosine and R represents the incorporation of A or G into the oligonucleotide.

The PCR reaction was carried out with TAKARA Taq polymerase following the manufacturer's instructions. The template used was 0.3 µg of the cDNA. The PCR program was:

1 cycle:	94 degrees	1 min
5 cycles:	94 degrees	4 sec
	50 degrees	30 sec
	72 degrees	1 min
5 cycles:	94 degrees	4 sec
	48 degrees	30 sec
	72 degrees	1 min
25 cycles:	94 degrees	4 sec
	46 degrees	30 sec
	72 degrees	1 min
1 cycle:	72 degrees	30 min

The fragment was purified by means of NucleoSpin Extract (Machery and Nagel) and cloned into the vector pGEMT (Promega) following the manufacturer's instructions.

The correctness of the fragment was checked by sequencing.

Example 3: Generation of a plant transformation construct for overexpressing HPPD with optimized DNA sequence (HPPDop) and elimination of HGD

To generate plants which express HPPDop in seeds and in which the expression of the endogenous HGD is suppressed by means of antisense technology, a binary vector was constructed which contains both gene sequences (Figure 2, construct VI).

a) Generation of an HPPDop expression cassette

To this end, the components of the cassette for expressing the HPPDop, composed of the legumin B promoter (accession No. X03677), the transit peptide of the spinach ferredoxin:NADP+ oxidoreductase (FNR; Jansen, T, et al (1988) Current Genetics 13, 517-522) and the NOS terminator (contained in pBI101 accession No. U12668) were first provided with the necessary restriction cleavage sites by means of PCR.

The legumin promoter was amplified by means of PCR from the plasmid plePOCS (Bäumlein, H, et al.(1986) Plant J. 24, 233-239) with the upstream oligonucleotide:

GAATTCGATCTGTCGTCTCAAACTC (SEQ ID NO: 4)

and the downstream oligonucleotide:

GGTACCGTGATAGTAAACAACTAATG (SEQ ID NO: 5)

and cloned into the vector PCR-Script (Stratagene) following the manufacturer's instructions.

The transit peptide was amplified from the plasmid pSK-FNR (Andrea Babette Regierer "Molekulargenetische Ansätze zur Veränderung der Phosphat-Nutzungseffizienz von höheren Pflanzen" [Approaches in molecular genetics for altering the phosphate utilization efficacy of higher plants], P+H Wissenschaftlicher Verlag, Berlin 1998 ISBN: 3-9805474-9-3) by means of PCR using the 5' oligonucleotide:

ATGGTACCTTTTTTGCATAAACTTATCTTCATAG (SEQ ID NO: 6)

and the 3' oligonucleotide:

ATGTCGACCCGGGATCCAGGGCCCTGATGGGTCCCATTTTCCC (SEQ ID NO: 7).

The NOS terminator was amplified from the plasmid pBI101 (Jefferson, R.A., et al (1987) EMBO J. 6 (13), 3901-3907) by means of PCR using the 5' oligonucleotide:

GTCGACGAATTTCCCCGAATCGTTC: (SEQ ID NO: 8)

and the 3' oligonucleotide

AAGCTTCCGATCTAGTAACATAGA (SEQ ID NO: 9).

The amplicon was cloned in each case into the vector pCR-Script (Stratagene) following the manufacturer's instructions.

For the expression cassette, the NOS terminator was first recloned as an SalI/HindIII fragment into a suitably cut pUC19 vector (Yanisch-Perron, C., et al (1985) Gene 33, 103-119). The transit peptide was subsequently introduced into this plasmid as an Asp718/SalI fragment. The legumin promoter was then cloned in as an EcoRI/Asp718 fragment. The gene HPPDop was introduced into this construct as an SalI fragment (Figure 3, construct III).

The finished cassette in pUC19 was used as template for a PCR, for which purpose the oligonucleotide:

AAGCTTGATCTGTCGTCTCAAACTC (SEQ ID NO: 10)

was used for the legumin promoter and the oligonucleotide:

AAGCTTCCGATCTAGTAACATAGA (SEQ ID NO: 11)

for the NOS terminator. The amplicon was cloned into pCR-Script and named pCR-ScriptHPPDop (Figure 3, construct IV).

b) Construction of an antiHGD expression cassette

To eliminate HGD using the antisense technique, the gene fragment was cloned as an SalI/Asp718 fragment into the vector pBinAR (Höfgen, R. and Willmitzer, L., (1990) Plant Sci. 66: 221-230) in which the 35S promoter and the OCS terminator are present (Figure 4, construct I). The construct acted as template for a PCR reaction with the oligonucleotide:

ATTCTAGACATGGAGTCAAAGATTCAAATAGA (SEQ ID NO: 12),

specifically for the 35S promoter sequence,
and the oligonucleotide:

ATTCTAGAGGACAATCAGTAAATTGAACGGAG (SEQ ID NO: 13).

specifically for the OCS terminator sequence

The amplicon was cloned into the vector PCR-Script (Stratagene) and named HGDanti (Figure 3, construct II).

c) Construction of the binary vector

To construct a binary vector for the transformation of oilseed rape, the construct HGDanti from pCRScriptHGDanti was first cloned into the vector pPTV (Becker, D., (1992) PMB 20, 1195-1197) as an XbaI fragment (Figure 5, construct V). The construct LegHPPDop from pCRScriptHPPDop was inserted into this plasmid as an HindIII fragment. This plasmid was termed pPTVHPPD/HGDanti (Figure 2, construct VI).

Example 4: Construction of cotransformation constructs for overexpressing HPPDop and eliminating HGD in *Brassica napus* plants

To cotransform plants with HPPDop and antiHGD, the construct legumin B promoter/transit peptide/HPPDop/NOS was excised from the vector pCRScriptHPPDop (Figure 3, construct IV) as an HindIII fragment and inserted into the suitably cut vector pPZP200 (Hajdukiewicz, P., et al., (1994) PMB 25(6): 989-94) (Figure 5, construct VII). This plasmid was used later for cotransforming plants together with the vector pPTVHGDanti (Figure 5, construct V) of Example 3 c).

Example 5: Generation of transgenic *Brassica napus* plants

The generation of transgenic oilseed rape plants approximately followed a protocol by Bade, J.B. and Damm, B. (in Gene Transfer to Plants, Potrykus, I. and Spangenberg, G., Hrsg., Springer Lab Manual, Springer Verlag, 1995, 30-38), which also indicates the composition of the media and buffers used.

The transformation was carried out with the *Agrobacterium tumefaciens* strain EHA105 (Li, X.Q., et al., PMB (1992) 20, 1037). Either the abovementioned plasmid pPTVHPPDopHGDanti (Figure 2) or cultures of agrobacteria with the plasmids pPTVHGDanti and pPZP200HPPDop (Figure 5), which cultures had been mixed after growing, were used for the transformation.

Seeds of *Brassica napus* var. Westar were surface-sterilized with 70% ethanol (v/v), washed in water for 10 minutes at 55°C, incubated for 20 minutes in a 1% strength hypochlorite solution (25% v/v Teepol, 0.1% v/v Tween 20) and washed six times for 20 minutes with sterile water. The seeds were dried for three days on filter paper and 10-15 seeds were germinated in a glass flask containing 15 ml of germination medium. The roots and apices were removed from several seedlings (approximate size 10 cm) and the remaining hypocotyls were cut into sections

approximately 6 mm in length. The approximately 600 explants thus obtained were washed for 30 minutes in 50 ml of basal medium and transferred into a 300-ml flask. After 100 ml of callus induction medium had been added, the cultures were incubated for 24 hours at 100 rpm.

Overnight cultures of the *Agrobacterium* strains were set up at 29°C in Luria broth medium supplemented with kanamycin (20 mg/l), of which 2 ml were incubated for 4 hours at 29°C in 50 ml of Luria broth medium without kanamycin until an OD_{600} of 0.4-0.5 had been reached. After the culture had been pelleted for 25 minutes at 2000 rpm, the cell pellet was resuspended in 25 ml of basal medium. The bacterial concentration in the solution was brought to an OD_{600} of 0.3 by adding more basal medium. For the cotransformation, equal portions of the solution of both strains were mixed.

The callus induction medium was removed from the oilseed rape explants by means of sterile pipettes, 50 ml of *Agrobacterium* solution were added, everything was mixed carefully and the mixture was incubated for 20 minutes. The *Agrobacterium* suspension was removed, the oilseed rape explants were washed for 1 minute with 50 ml of callus induction medium, and 100 ml of callus induction medium were subsequently added. Cocultivation was carried out for 24 hours on an orbital shaker at 100 rpm. Cocultivation was stopped by removal of the callus induction medium, and the explants were washed twice for 1 minute in each case with 25 ml and twice for 60 minutes with in each case 100 ml of wash medium at 100 rpm. The wash medium together with the explants were transferred into 15-cm Petri dishes and the medium was removed using sterile pipettes.

For the regeneration, in each case 20-30 explants were transferred into 90-mm Petri dishes containing 25 ml of shoot induction medium supplemented with phosphinotricin. The Petri dishes were sealed with 2 layers of Leukopor and incubated at 25°C and 2000 lux at photoperiods of 16 hours light/8 hours darkness. Every 12 days, the calli which developed were transferred to fresh Petri dishes with shoot induction medium. All further steps for the regeneration of entire plants were carried out as described by Bade, J.B and Damm, B. (in: Gene Transfer to Plants, Potrykus, I. and Spangenberg, G., Hrsg., Springer Lab Manual, Springer Verlag, 1995, 30-38).

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